Journal of Food Protection, Vol. 72, No. 1, 2009, Pages 85–92

Control of Foodborne Pathogens and Soft-Rot Bacteria on Bell Pepper by Three Strains of Bacterial Antagonists†

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MS 08-268: Received 9 June 2008/Accepted 31 July 2008

ABSTRACT

Forty-two representative strains of native bacteria associated with fresh peeled baby carrots were isolated and characterized. Two of these strains, identified as *Pseudomonas fluorescens* AG3A (Pf AG3A) and *Bacillus* YD1, were evaluated in conjunction with another known antagonist, *P. fluorescens* 2-79 (Pf 2-79), for their potential as biocontrol agents of human pathogens (*Listeria monocytogenes, Yersinia enterocolitica, Salmonella enterica,* and *Escherichia coli* O157:H7) and soft-rot bacteria (*Erwinia carotovora* subsp. *carotovora, Pseudomonas marginalis,* and *Pseudomonas viridiflava*). When grown on iron-deficient agar media, all three antagonists produced inhibition zones up to 25 mm in diameter against the growth of human pathogens and soft-rot bacteria. However, when grown on iron-rich agar media, only Pf 2-79 and *Bacillus* YD1 exhibited antimicrobial activity. Treatment of bell pepper disks with Pf 2-79 or *Bacillus* YD1 reduced the growth of pathogen by 1.4 to 4.1 log units, depending upon the ratio of the number of antagonist cells to pathogen cells (1:1, 10:1, 100:1, or 1,000:1). The greatest reduction was observed when 10- to 100-fold higher number of antagonists than pathogens was applied. Pf AG3A and *Bacillus* YD1 reduced the growth of pathogens on pepper disks at 20°C but not at 10°C. However, Pf 2-79 reduced the growth of *L. monocytogenes* and *Y. enterocolitica* by up to 4 log units at either 20 or 10°C. Treatment of pepper disks with Pf 2-79 also reduced the incidence of soft rot induced by soft-rot bacteria by 40 to 70%. Pf 2-79 is the most effective of the three antagonists tested for control of spoilage bacteria and human pathogens on bell pepper.

During the last two decades, an increasing number of foodborne illness outbreaks have been associated with the consumption of fresh produce *(26).* In spite of extensive testing, conventional washing and sanitization treatments are unable to remove the pathogens from contaminated produce to an acceptable level *(22).* More effective control strategies are needed to meet the 5-log pathogen reduction target as recommended by the U.S. Food and Drug Administration *(19).* Such strategies may include the use of biological interventions to suppress the growth of survivor pathogens after chemical or physical treatment. A wide variety of biological products currently are available for control of phytopathogens. For example, *Agrobacterium radiobacter* K84 has been used to control crown gall pathogen *Agrobacterium tumefaciens (21),* and *Lactobacillus reuteri* has been used to control *Salmonella* in newly hatched chicks *(24).* However, research on the development of biological agents for control of human pathogens on fresh produce has not been fully explored.

Indigenous microorganisms present on the surfaces of plants can inhibit or enhance the survival or growth of human pathogens on fresh produce *(20).* Babic et al. *(1)* reported that native bacteria associated with fresh-cut spinach restricted the growth of *Listeria monocytogenes,* and Carlin et al. *(2)* found that *L. monocytogenes* grew more rapidly on endive leaves that had been treated with 10% hydrogen peroxide to reduce the number of background microflora. Francis and O'Beirne *(5)* found that native microflora associated with lettuce inhibited the growth of *Listeria* species on the surfaces of shredded lettuce. Recently, Liao *(13)* reported that the native microbial complex recovered from fresh peeled baby carrots inhibited the growth of pathogens by 2 to 3 log units both in vitro and in situ *(13).* Resident microorganisms present on the surfaces of fresh produce thus provide a rich source for isolation of individual strains of antagonists for control of foodborne pathogens.

Various biotypes of *Pseudomonas fluorescens* constitute a major component of native bacteria associated with fresh and minimally processed produce, including endive *(2),* spinach *(1),* and lettuce *(5).* Some of these fluorescent pseudomonads have been effective for reducing the growth of plant and/or human pathogens *(8, 12).* One such strain, *P. fluorescens* 2-79, has been studied for many years as a biocontrol agent of the fungal pathogen *Gaeumannomyces graminis* var. *tritici* that attacks wheat *(9).* This pseudomonad also was antagonistic to *Salmonella enterica* in vitro and on seed sprouts *(4).* The objectives of this study were to (i) isolate and characterize representative strains of native bacteria associated with fresh peeled baby carrots, (ii) identify individual strains that are antagonistic to major foodborne pathogens and soft-rot bacteria by agar spot bioassays, and (iii) evaluate the potential of two newly identified antagonists (*P. fluorescens* AG3A and *Bacillus* sp. YD1) and the known antagonist *P. fluorescens* 2-79 as biocontrol agents for foodborne pathogens and soft-rot bacteria on bell pepper slices.

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TABLE 1. *Bacterial strains used*

Bacterium	Strain	Phenotype	Origin
Human pathogens			
Listeria monocytogenes	Scott A	Wild type	J. Call, ERRC[a]
Yersinia enterocolitica	JB580V	Nalidixic acid resistant[b]	V. Miller, Washington University, St. Louis, Mo.
Salmonella enterica Mbandaka	S14	Nalidixic acid resistant	C.-H. Liao, ERRC
Escherichia coli O157:H7	13B 88	Nalidixic acid resistant	B. Annous, ERRC
Soft-rot bacteria			
Pseudomonas marginalis	BC-05-1B	Nalidixic acid resistant	C.-H. Liao, ERRC
Erwinia carotovora subsp. *carotovora*	SR319	Nalidixic acid resistant	A. Kelman, North Carolina State University
P. viridiflava	PJ-08-6A	Nalidixic acid resistant	C.-H. Liao, ERRC
Antagonists			
P. fluorescens	2-79	Rifampin resistant[b]	W. Fett, ERRC
P. fluorescens	AG3A	Rifampin resistant	This study
Bacillus sp.	YD1	Chloramphenicol resistant[b]	This study

[a] ERRC, Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, Wyndmoor, Pa.
[b] Resistant to 100 μg/ml nalidixic acid, 100 μg/ml rifampin, or 500 μg/ml chloramphenicol.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The four strains of foodborne bacterial pathogens, three strains of soft-rot bacteria, and three strains of bacterial antagonists used in this study are listed in Table 1. With the exception of *L. monocytogenes,* antibiotic-resistant derivatives of pathogens, soft-rot bacteria, and antagonists were isolated via spontaneous mutation and used throughout the study. Tryptic soy agar (TSA) supplemented with 100 μg/ml nalidixic acid was used to culture and enumerate nalidixic acid–resistant derivatives of pathogens and soft-rot bacteria. *Pseudomonas* agar F (KB; Difco, Becton Dickinson, Sparks, Md.) supplemented with 100 μg/ml rifampin was used to culture and enumerate the rifampin-resistant derivatives of *P. fluorescens* and *Pseudomonas marginalis. Bacillus* YD1was recovered from produce by plating appropriately diluted samples onto TSA supplemented with 500 μg/ml chloramphenicol. *L. monocytogenes* was routinely cultured on TSA but recovered from inoculated produce by plating appropriately diluted samples onto PALCAM agar (Difco, Becton Dickinson). Cultures plates for enumeration of pathogens were incubated at 37°C for 24 to 48 h, whereas those for enumeration of soft-rot bacteria or antagonists were incubated at 28°C for 2 days. When a liquid medium was needed, tryptic soy broth supplemented with appropriate antibiotics was used.

Isolation and characterization of native bacteria. Fresh peeled baby carrots were purchased at local supermarkets. Ten pieces of baby carrot (approximately 50 g) were placed in a Stomacher bag containing 100 ml of phosphate-buffered saline (PBS). Prepared PBS (75 μM, pH 7.2, containing 15.4 μM KH_2PO_4, 1.6 mM NaCl, and 27.1 μM $Na_2{\cdot}HPO_4$; Life Technologies, Inc., Rockville, Md.) was used throughout the study. The bag containing baby carrots and PBS was pummeled at high speed for 2 min in a laboratory Stomacher (Seward Medical Ltd., London, UK). Ten-fold serially diluted homogenates were then spread plated onto TSA and subsequently incubated at 28°C for 2 days. Forty-two representative strains of native bacteria, each displaying a distinct colony phenotype, were isolated and characterized. Microbiological properties, including Gram staining, sporulation, motility, gliding ability, oxidase and catalase activities, and accumulation of poly-β-hydroxybutyrate, were determined using the standard methods as previously described *(7, 23).* Gram-negative aerobic rods also were tested for their ability to grow on a selective cetrimide, fucidine, and cephaloridine (CFC) agar *(17)* developed specifically for detection of *Pseudomonas.* Biochemical characterization and carbohydrate utilization pattern were determined using the API 20E kit (bioMérieux, Inc., Hazelwood, Mo.) for identification of *Enterobacter* and *Serratia* and the API 20NE kit for identification of *Pseudomonas* and other nonenteric rods. *Flavobacterium* and *Cytophaga* strains were identified primarily based on their ability to produce orange or yellow pigmentation and their gliding ability. Pectolytic activity was determined by analyzing the ability of each strain to grow and form a depression on a semisolid pectate medium *(16).* The identity of two newly characterized antagonists, designated *P. fluorescens* AG3A (Pf AG3A) and *Bacillus* YD1, was further confirmed by 16S rRNA gene sequencing (Microbial ID, Inc., Newark, Del.).

Analysis of antimicrobial activity by agar spot bioassays. Forty-two representative strains of native bacteria isolated as described above were tested for their inhibitory activity against *L. monocytogenes* by agar spot bioassay *(15).* Each strain was spotted onto a KB plate and then incubated at 28°C for 3 days. After incubation, culture plates were first exposed to chloroform vapor for 30 min to kill the testing bacterium grown in the center of the agar plate and then overlaid with 3 ml of water agar (0.6%) containing approximately 10^5 CFU/ml *L. monocytogenes.* A positive antagonistic reaction was indicated by the formation of a clear inhibition zone (≥1.5 mm in diameter) of *L. monocytogenes* growth after incubation of culture plates at 37°C for 2 days. The inhibition zone was measured from the edge of the colony of a testing bacterium grown in the center to the perimeter of the clear inhibition zone. Two isolates designated as Pf AG3A and *Bacillus* YD1 were further investigated in conjunction with *P. fluorescens* 2-79 (Pf 2-79) for their ability to inhibit the growth of other pathogens and soft-rot bacteria on three agar media: TSA, KB, and KB supplemented with 3 mM $FeCl_3$ (KB-Fe^{3+}).

Preparation of mixed bacterial suspensions containing pathogen and antagonist in different ratios. Four foodborne pathogens, *S. enterica, Yersinia enterocolitica, L. monocytogenes,* and *Escherichia coli* O157:H7, were each grown on TSA supple-

mented with appropriate antibiotic and incubated at 37°C for 18 h. Pf 2-79 and Pf AG3A were grown on KB supplemented with 100 μg/ml rifampin at 28°C for 2 days. *Bacillus* YD1 was grown on TSA supplemented with 500 μg/ml chloramphenicol at 37°C for 18 h. Immediately after incubation, an aliquot of bacterial mass on the agar plate was harvested with a sterile inoculation loop and suspended in PBS by vigorous shaking to an optical density of approximately 1.0 at 600 nm (equivalent to approximately 2×10^9 CFU/ml). A series of bacterial suspensions containing a constant number of pathogen cells (approximately 10^5 CFU/ml) and a 10-fold increasing number of antagonist cells (10^5, 10^6, 10^7, or 10^8 CFU/ml) were prepared by mixing an equal volume of two bacterial suspensions containing either a pathogen or an antagonist to make mixtures in which the ratio of pathogen to antagonist was 1:1, 1:10, 1:100, or 1:1,000.

Inoculation and recovery of foodborne pathogens from bell pepper disks. Green bell pepper disks have been used as a model to study bacterial attachment and the response of attached bacteria to chemical and biological treatments *(13, 14).* Bell pepper disks prepared as described were uniform in size and shape and contained a consistent injured and noninjured surface area. The number of bacteria that would attach to the disks was mainly dependent on the bacterial density in the suspension used for immersion. The number of bacteria attached to pepper disks following immersion was predictable and consistent from experiment to experiment *(13).* Thus, bell pepper disks were used as a model in this study to evaluate three antagonists (Pf AG3A, Pf 2-79, and *Bacillus* YD1) for their use as biocontrol agents for pathogens and soft-rot bacteria in situ. Green bell peppers purchased at a local grocer were first cleaned with a laboratory detergent (Liquid-Nox, Alconox, Inc., White Plains, N.Y.) and then rinsed with 80% (vol/vol) ethanol and sterile water. Pepper disks (approximately 1 g per disk) were prepared using a sterile cork borer (no. 9, with a 154-mm^2 cross section) to excise sections from different parts of the fruit as previously described *(14).* After preparation, pepper disks were pooled, and 30 disks were randomly selected and immersed for 2 min in a bacterial suspension containing a pathogen (*S. enterica, Y. enterocoltica, E. coli* O157:H7, and *L. monocytogenes*) at approximately 10^5 CFU/ml and a 10-fold increasing level of an antagonist (10^5, 10^6, 10^7, or 10^8 CFU/ml). After immersion, pepper disks were placed in six stomacher bags (five disks per bag). Three bags each were removed and used as a composite sample to determine the change in the pathogen level on pepper disks before and after incubation at 20°C for 2 days. For determination of the pathogen level on pepper disks in each bag, 50 ml of PBS was added to the bag, which was then pummeled at high speed for 2 min in a laboratory stomacher. Ten-fold serially diluted homogenates were then spread plated onto PALCAM agar for isolation of *L. monocytogenes* or onto TSA supplemented with 100 μg/ml nalidixic acid for isolation of other pathogens.

Analysis of the effect of antagonists on the growth of pathogens on pepper disks stored at refrigeration temperature. Because fresh produce products usually are stored at 10°C or below, a series of experiments were conducted to determine whether the growth of two cold-tolerant pathogens (*L. monocytogenes* and *Y. enterocolitica*) on refrigerated pepper disks could be reduced by treating the disks with Pf 2-79. A total of 300 pepper disks prepared as described above were pooled and randomly divided into four lots. Each lot of 75 disks was immersed in a bacterial suspension containing either *L. monocytogenes* or *Y. enterocolitica* (approximately 10^5 CFU/ml) in the presence or absence of Pf 2-79 (approximately 10^7 CFU/ml). After immersion, the disks from each lot were placed in 15 stomacher bags (five disks per bag) and incubated at 10°C for 8 days. The population of *L. monocytogenes* or *Y. enterocolitica* on pepper disks coinoculated with or without Pf 2-79 was determined at 2-day intervals. Three bags at each interval (days 0, 2, 4, 6, and 8) were removed, and the pepper disks were used to determine the change in the number of either pathogen during storage. By following the same protocol, pepper disks inoculated with *Salmonella* or *E. coli* O157:H7 alone or in combination with one of three antagonists were prepared and used to monitor the change in the population of either pathogen during storage at 10°C for 8 days.

Analysis of the effect of Pf 2-79 on the growth of soft-rot bacteria on pepper disks. A total of 180 pepper disks prepared as described above were pooled and randomly divided into six lots. Each lot consisting of 30 disks was immersed for 2 min in a *P. marginalis, Pseudomonas viridiflava,* or *Erwinia carotovora* subsp. *carotovora* suspension containing approximately 10^5 CFU/ml in the presence or absence of approximately 10^7 CFU/ml Pf 2-79. A separate lot consisting of 15 disks was immersed for 2 min in each suspension containing a given level of antagonists and soft-rot bacteria and used to evaluate the effect of Pf 2-79 on the development of soft rot. After immersion, each lot inoculated with a given soft-rot bacterium in the presence or absence of Pf 2-79 was placed in six stomacher bags (five disks per bag). Three bags from each lot were removed, and the pepper disks were used to determine the change in the level of soft-rot bacteria with or without Pf 2-79 and before or after incubation at 10°C for 8 days. Recovery of *E. carotovora, P. viridiflava,* or *P. marginalis* from pepper disks was conducted using a procedure similar to that described for recovery of pathogens. Appropriately diluted homogenates were spread plated onto TSA supplemented with 100 μg/ml nalidixic acid. The number of disks showing soft rot was recorded after incubation at 10°C for 8 days, and the data obtained were used to calculate the percent incidence of soft rot.

Statistical analyses. The changes in the pathogen population on pepper disks as affected by the variation in the ratio of antagonist versus pathogen were analyzed with an analysis of variance. The change in the population of *L. monocytogenes* or *Y. enterocolitica* on pepper disks treated with or without Pf 2-79 also was analyzed with an analysis of variance. Differences between treatments were analyzed using the Bonferroni least significant difference mean separation procedure *(18)* at the $P = 0.05$ significance level.

RESULTS

Characterization of native bacteria associated with baby carrot and selection of strains antagonistic to *L. monocytogenes*. Microbiological and biochemical properties of 42 representative strains of native bacteria isolated from baby carrot were characterized using the standard procedures previously described *(7, 23).* These strains were divided into five taxonomic groups based on a number of properties (described in ''Materials and Methods''). A majority (36 of 42 strains) were identified as aerobic gram-negative rods, and 50% of these rods were identified as *Pseudomonas* based on their ability to produce fluorescent pigments. Nine strains were identified as *Enterobacter* or *Serratia,* which could be readily differentiated from *Pseudomonas* by their inability to grow on a diagnostic CFC agar *(17).* Three strains of *Flavobacterium* or *Cytophaga* were identified primarily based on their ability to produce

TABLE 2. *In vitro inhibition of foodborne pathogens and soft rot bacteria by* Pseudomonas fluorescens *2-79,* Pseudomonas fluorescens *AG3A, and* Bacillus *YD1 on* Pseudomonas *agar F (KB), KB supplemented with 3 mM $FeCl_3$ (KB-Fe^{3+}), and tryptic soy agar (TSA)*

Targeted pathogens and spoilage bacteria	Growth inhibition zone (mm diam) caused by three antagonists[a]:								
	P. fluorescens 2-79			*P. fluorescens* AG3A			*Bacillus* YD1		
	KB	KB-Fe^{3+}	TSA	KB	KB-Fe^{3+}	TSA	KB	KB-Fe^{3+}	TSA
Yersinia enterocolitica	21	15	20	22	ND	ND	9	6	11
Listeria monocytogenes	20	21	17	33	ND	ND	16	16	11
Salmonella enterica	15	23	22	15	ND	ND	4	5	6
Escherichia coli O157:H7	25	25	15	22	5	ND	5	10	7
Pseudomonas marginalis	16	ND	ND	20	ND	ND	11	13	4
Erwinia carotovora subsp. *carotovora*	16	0	15	17	ND	ND	4	11	7
P. viridiflava	18	15	11	24	ND	ND	7	6	12

[a] Each value represents the mean of four determinations; the clear zone was measured from the edge of the colony of the antagonistic bacterium to the perimeter of the lawn of the pathogen or soft-rot bacterium. ND, not detectable.

orange or yellow pigments and their gliding ability. Five of the 42 strains were identified as gram-positive rods: one strain of lactic acid bacteria and four strains of *Bacillus.* Pectolytic activity was detected in four strains of *Pseudomonas* and one strain each of *Enterobacter* and *Cytophaga.*

The 42 strains of native bacteria isolated also were tested by agar spot bioassay for their ability to inhibit the growth of *L. monocytogenes.* Eight strains of fluorescent pseudomonads and two strains of *Bacillus* sp. produced an inhibition zone ranging from 3 to 20 mm in diameter against the growth of *L. monocytogenes* on KB. One *P. fluorescens* isolate (Pf AG3A) and one *Bacillus* isolate (*Bacillus* YD1) exhibiting the highest degree of inhibition against *L. monocytogenes* were selected for further investigation.

Analysis of antimicrobial activity of three bacterial antagonists by agar spot bioassay. Pf AG3A and *Bacillus* YD1 were investigated in conjunction with another antagonist, Pf 2-79, for their inhibitory activity against pathogens (*L. monocytogenes, Y. enterocolitica, S. enterica,* and *E. coli* O157:H7) and soft-rot bacteria (*P. marginalis, E. carotovora,* and *P. viridiflava*) on three different agar media (KB, KB-Fe^{3+}, and TSA). Pf 2-79 was the most effective and Pf AG3A the least effective among the three antagonists tested in producing the inhibition zones against the growth of pathogens and soft-rot bacteria on three agar media examined (Table 2). Pf AG3A exhibited antimicrobial activity only when it was grown on iron-deficient KB medium. No inhibition against pathogens or soft-rot bacteria was observed when Pf AG3A was grown on iron-rich TSA or KB-Fe^{3+} media. Both Pf 2-79 and *Bacillus* YD1 displayed anti-*Listeria* activity when grown on iron-deficient KB or on iron-rich TSA or KB-Fe^{3+} media. This finding indicates that the antimicrobial activity of Pf 2-79 and *Bacillus* YD1 was not simply due to the production of iron-chelating siderophores *(6, 11)* under iron-deficient conditions but also was due to the production of other antimicrobials, e.g., the production of phenazine carboxylic acid by Pf 2-79 under iron-rich conditions *(9).*

Inhibition of pathogens by three antagonists on pepper disks as affected by the ratio of antagonist to pathogen. Pepper disks were immersed for 2 min in a bacterial suspension containing a constant level (approximately 10^5 CFU/ml) of a pathogen (*Y. enterocolitica, L. monocytogenes, S. enterica,* or *E. coli* O157:H7) and a 10-fold increasing level (approximately 10^5, 10^6, 10^7, or 10^8 CFU/ml) of *Bacillus* YD1. The number of pathogen cells attached to pepper disks immediately after immersion was determined to be 3.2 to 3.8 log CFU per disk. After incubation at 20°C for 2 days, the number of pathogen cells on pepper disks not treated with YD1 increased from the initial 3.2 to 3.8 to a final 8.3 to 8.8 log CFU per disk. On pepper disks treated with YD1, the growth of pathogen was reduced by 1.4 to 3.8 log CFU, depending on the ratio of YD1 to pathogen (1:1, 10:1, 100:1, or 1,000:1) (Table 3). A significantly greater reduction ($P \leq 0.05$) was observed as higher levels of antagonist were used.

On pepper disks treated with Pf 2-79, the growth of pathogens also was greatly reduced. A positive correlation was observed between the degree of growth reduction and the ratio of Pf 2-79 to pathogen (Table 4). The greatest reduction was observed on pepper disks coinoculated with Pf 2-79 at 5.7 to 6.7 log CFU per disk and pathogen at 3.4 to 3.7 log CFU per disk. In general, application of 10- to 100-fold higher levels of YD1 or Pf 2-79 than pathogen was required to achieve a maximal growth reduction of 3 to 4 log CFU.

Pf AG3A was the least effective among the three antagonists tested for reducing the growth of pathogens on pepper disks. No growth reduction was observed on pepper disks treated with approximately the same level or 10-fold higher level of Pf AG3A. However, on pepper disks treated with 100- or 1,000-fold higher levels of Pf AG3A, the growth of pathogen was reduced by 0.8 to 1.5 log CFU, which was a significant reduction ($P \leq 0.05$) compared with the growth on untreated disks. All four strains of pathogens grew well on pepper disks not treated with antagonists, and the final population of each pathogen increased 4 to 5 log CFU after incubation of disks at 20°C for 2 days

TABLE 3. *Inhibition of* Yersinia enterocolitica, Listeria monocytogenes, Salmonella enterica*, and* Escherichia coli *O157:H7 on bell pepper disks by 10-fold increasing levels of* Bacillus *YD1 after incubation of inoculated disks at 20°C for 2 days*

Ratio of *Bacillus*:pathogen[a]	Final population (log CFU/disk) of pathogen (log reduction)[b]			
	Y. enterocolitica	*L. monocytogenes*	*S. enterica*	*E. coli* O157:H7
0:1 (control)	8.6 ± 0.3[b] A	8.3 ± 0.4 A	8.8 ± 0.2 A	8.5 ± 0.3 A
1:1	7.2 ± 0.4 B (1.4)	5.8 ± 0.6 B (2.5)	6.7 ± 0.3 B (2.1)	6.6 ± 0.3 B (1.9)
10:1	6.8 ± 0.4 B (1.8)	5.5 ± 0.1 B (2.8)	6.0 ± 0.2 C (2.8)	6.3 ± 0.5 B (2.2)
100:1	5.3 ± 0.2 C (3.3)	5.1 ± 0.3 C (3.2)	5.0 ± 0.5 D (3.8)	4.7 ± 0.3 C (3.8)
1,000:1	5.0 ± 0.3 C (3.6)	4.9 ± 0.5 C (3.4)	5.2 ± 0.3 D (3.6)	5.2 ± 0.1 C (3.3)

[a] The average level of *Bacillus* YD1 on pepper disks ($n = 6$) before incubation was estimated to be 3.5, 4.5, 5.5, and 6.5 log CFU per disk, respectively. The average pathogen level on pepper disks before incubation was determined to be 3.2, 3.5, 3.6, and 3.8 log CFU per disk for *E. coli* O157:H7, *L. monocytogenes, Y. enterocolitica*, and *S. enterica*, respectively.

[b] Values represent the mean ± standard deviation of six determinations from two experiments and three replications ($n = 6$). Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$). Disks inoculated with the pathogen alone were used as controls for calculation of the log reduction.

(Tables 3 and 4). Regardless of the initial concentration of antagonists on pepper disks (3 to 5 log CFU per disk), the final populations reached a maximum of approximately 9 log CFU per disk after incubation (data not shown).

Inhibition of pathogens by three antagonists on pepper disks stored at 10°C. On pepper disks not treated with Pf 2-79, the population of *L. monocytogenes* and *Y. enterocolitica* increased from the initial 3 to 4 log CFU per disk to more than 7 log CFU per disk after incubation of disks at 10°C for 8 days. However, on pepper disks treated with Pf 2-79 at 5 to 6 log CFU per disk, the population of *L. monocytogenes* or *Y. enterocolitica* showed very little increase after incubation of disks at 10°C for 8 days. Inoculation of pepper disks with a 100-fold higher level of Pf 2-79 than pathogen almost completely inhibited the growth of *L. monocytogenes* and *Y. enterocolitica* on refrigerated pepper disks (Fig. 1). However, treatment of pepper disks with *Bacillus* YD1 did not restrict the growth of *L. monocytogenes* and *Y. enterocolitica,* primarily because of the inability of this antagonist to grow on pepper disks that were stored at low temperature. Although Pf AG3A grew on the disks to a density of approximately 9 log CFU per disk, treatment of pepper disks with this antagonist reduced the growth of *L. monocytogenes* or *Y. enterocolitica* by only 0.4 log CFU; this final pathogen population was not significantly different ($P \leq 0.05$) from that on untreated disks. The populations of *Salmonella* and *E. coli* O157:H7 on pepper disks treated with or without Pf 2-79 remained unchanged during storage at 10°C for 8 days, indicating no effect of Pf 2-79 on the survival of these two pathogens on refrigerated pepper disks (data not shown).

Inhibition of soft-rot bacteria by Pf 2-79 on pepper disks stored at 10°C. Inoculation of pepper disks with Pf 2-79 greatly reduced the growth of soft-rot pseudomonads, including *P. marginalis* and *P. viridiflava,* on pepper disks stored at 10°C for 8 days (Table 5). On pepper disks not inoculated with Pf 2-79, the population of two soft-rot pseudomonads had a net increase of 4.4 to 4.5 log CFU after incubation of pepper disks at 10°C for 8 days. However, on pepper disks inoculated with Pf 2-79 at 5 to 6 log CFU per disk, the populations of *P. marginalis* and *P. viridiflava* had a net increase of only 2.0 to 2.4 log CFU. Hence, treatment of pepper disks with Pf 2-79 could suppress the growth of soft-rot pseudomonads by 2.0 to 2.5

TABLE 4. *Inhibition of* Yersinia enterocolitica, Listeria monocytogenes, Salmonella enterica*, and* Escherichia coli *O157:H7 on bell pepper disks by 10-fold increasing levels of* Pseudomonas fluorescens *2-79 (Pf 2-79) after incubation of inoculated disks at 20°C for 2 days*

Ratio of Pf 2-79:pathogen[a]	Final population (log CFU/disk) of pathogen (log reduction)[b]			
	Y. enterocolitica	*L. monocytogenes*	*S. enterica*	*E. coli* O157:H7
0:1 (control)	8.7 ± 0.3[b] A	8.3 ± 0.4 A	8.5 ± 0.2 A	8.8 ± 0.4 A
1:1	6.6 ± 0.4 B (2.1)	5.4 ± 0.5 B (2.9)	6.2 ± 0.4 B (2.3)	6.3 ± 0.3 B (2.5)
10:1	5.0 ± 0.4 C (3.7)	5.1 ± 0.1 B (3.1)	5.6 ± 0.3 C (2.9)	6.0 ± 0.2 B (2.8)
100:1	5.2 ± 0.3 C (3.5)	4.5 ± 0.1 C (3.8)	4.4 ± 0.2 D (4.1)	5.2 ± 0.5 C (3.6)
1,000:1	4.9 ± 0.2 C (3.8)	4.3 ± 0.4 C (4.0)	4.7 ± 0.2 D (3.8)	4.9 ± 0.3 C (3.9)

[a] The average level of Pf 2-79 on pepper disks ($n = 6$) before incubation was estimated to be 3.7, 4.7, 5.7, and 6.7 log CFU per disk, respectively. The average pathogen level on pepper disks before incubation was determined to be 3.3, 3.5, 3.7, and 3.4 log CFU per disk for *E. coli* O157:H7, *L. monocytogenes, Y. enterocolitica*, and *S. enterica*, respectively.

[b] Values are the mean ± standard deviation of six determinations from two experiments and three replications ($n = 6$). Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$). Disks inoculated with the pathogen alone were used as controls for calculation of the log reduction.

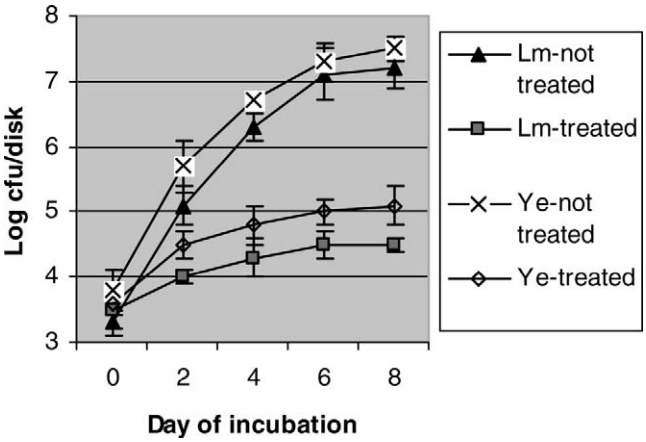

FIGURE 1. *Comparison of the growth of* Listeria monocytogenes *(Lm) and* Yersinia enterocolitica *(Ye) on bell pepper disks treated with or without* Pseudomonas fluorescens *2-79. Bell pepper disks containing* L. monocytogenes *or* Y. enterocolitica *at 10^3 to 10^4 CFU per disk were cultured with or without* P. fluorescens *2-79 at 10^5 to 10^6 CFU per disk. Inoculated pepper disks were incubated at 10°C for 8 days, and the change in the population of* L. monocytogenes *or* Y. enterocolitica *on pepper disks was determined at 2-day intervals.*

log CFU. Treatment of pepper disks with Pf 2-79 also reduced the incidence of soft rot caused by *P. marginalis* and *P. viridiflava* by 73% and reduced the incidence of soft rot caused by *E. carotovora* by 40%.

DISCUSSION

The native microbial complex associated with fresh peeled baby carrot can inhibit the growth of foodborne pathogens both in vitro and on bell pepper disks *(13).* The first objective of this study was to characterize representative strains of native bacteria associated with baby carrot and to identify strains that are antagonistic to human pathogens. Results indicated that fluorescent pseudomonads are a major component of the native bacteria associated with fresh peeled baby carrot. A large number of these pseudomonads (8 of 18 strains examined) exhibited anti-*Listeria* activity. Fluorescent pseudomonads as a group seem to play an important role in the antimicrobial activity of carrot-related microflora, as previously demonstrated *(13).* As common saprophytes, fluorescent pseudomonads, including *P. fluorescens, Pseudomonas putida,* and *Pseudomonas aeruginosa,* have been found closely associated with raw foods such as fish, meat, milk, and fresh produce *(8, 12, 20).* Some of these pseudomonads have been suggested as potential biocontrol agents, similar to Pf AG3A, for pathogens or spoilage bacteria on fish *(8),* sprouts *(4),* vegetables *(25),* potato *(15),* and apple *(10).* However, use of antagonistic pseudomonads alone did not completely eliminate pathogens but simply suppressed their growth *(12).*

A large proportion of fluorescent pseudomonads associated with produce are pectolytic and capable of producing an array of pectolytic enzymes for degradation of plant tissues *(16).* Those strains capable of producing cell wall–degrading enzymes generally are not considered candidates for biocontrol agents because of their potential for causing damage to produce. Four of 18 fluorescent pseudomonads isolated in this study also exhibited pectolytic activity. Although the detrimental effects of these pseudomonads on fresh produce has not been investigated, the risk associated with the application of an undefined microbial complex from baby carrot *(13)* or seed sprout *(4)* as a biocontrol agent cannot be ignored. All three antagonists examined in the study (Pf 2-79, Pf AG3A, and *Bacillus* YD1) did not produce pectolytic enzymes and other depolymerases. Therefore, these antagonists are not expected to have an adverse effect on tissue integrity. However, the effects on produce of these antagonists when applied in large quantities as biocontrol agents needs to be evaluated.

In the present study, Pf 2-79 was more effective than Pf AG3A and *Bacillus* YD1 for control of pathogens on fresh produce stored at refrigeration temperature. Pf 2-29 is a psychrotroph capable of growing on fresh produce at refrigeration temperatures. This pseudomonad is nutritionally versatile and capable of utilizing simple carbohydrates available in different types of produce as energy sources.

TABLE 5. *Inhibition of soft-rot bacteria (*Pseudomonas marginalis, Pseudomonas viridiflava*, and* Erwinia carotovora *subsp.* carotovora*) by* Pseudomonas fluorescens *2-79 on green bell pepper disks after incubation of inoculated disks at 10°C for 8 days*

Disks inoculant[a]	Population of soft-rot bacteria on pepper disks (log CFU/disk)[b]			Incidence of soft rot (among 15 disks tested)	
	Before incubation	After incubation	Log increase	No. of disks rotted	% of disks rotted
P. marginalis alone	4.1 ± 0.4	8.5 ± 0.3	4.4	15	100
P. marginalis + *P. fluorescens* 2-79	3.9 ± 0.3	6.3 ± 0.2	2.4	4	26.7
P. viridiflava alone	3.8 ± 0.2	8.3 ± 0.5	4.5	13	86.7
P. viridiflava + *P. fluorescens* 2-79	4.2 ± 0.4	6.2 ± 0.3	2.0	2	13.3
E. carotovora alone	4.4 ± 0.3	7.1 ± 0.3	2.7	6	40.0
E. carotovora + *P. fluorescens* 2-79	4.6 ± 0.1	5.9 ± 0.2	1.3	0	0

[a] Bell pepper disks were immersed for 2 min in a bacterial suspension containing approximately 10^5 CFU/ml *P. marginalis, P. viridiflava*, or *E. carotovora* with or without 10^7 CFU/ml *P. fluorescens* 2-79.

[b] Bacterial were recovered from pepper disks immediately after immersion and 8 days after incubation. Values for the bacterial population are the mean ± standard deviation of two experiments and three replicates ($n = 6$). Growth of each soft-rot bacterium was significantly reduced ($P \leq 0.05$) compared with growth on untreated disks.

Results (Fig. 1) indicate that growth of two cold-tolerant pathogens (*L. monocytogenes* and *Y. enterocolitica*) was almost completed inhibited on pepper disks coinoculated with Pf 2-79. Previously, Pf 2-79 inhibited the growth of *Salmonella* on sprout seeds *(4).* The results of the present study further indicate that Pf 2-79 may be used to suppress the growth of pathogens and soft-rot bacteria on bell pepper and possibly other types of produce.

The mechanism by which strains Pf 2-79, Pf AG3A, and *Bacillus* YD1 inhibit the growth of pathogens on pepper disks has not been determined. In other studies *(6, 9, 11),* the antimicrobial mechanism of fluorescent pseudomonads was mainly due to the production of iron-chelating siderophores under iron-deficient conditions. In the present study, most of the pseudomonads associated with baby carrot exhibited antimicrobial activity only when grown on KB medium but not on TSA or KB medium supplemented with $FeCl_3$. This finding indicates that the antimicrobial activity of those pseudomonads examined in this study is primarily due to the production of iron-chelating siderophores. Because Pf 2-79 and *Bacillus* YD1 exhibited antimicrobial activity when grown on agar media either rich or deficient in ferric ions, the antimicrobial action of these two antagonists may be due to the production of both iron-chelating siderophores and other antimicrobials. In addition to iron-chelating siderophores, Pf 2-79 can produce an antimicrobial identified as phenazine carboxylic acid *(9).* A biocontrol strain of *Bacillus* also can produce an ''iturin''-like antimicrobial and possibly other antimicrobials not related to chelating siderophores *(3).* Further study is needed to determine whether phenazine carboxylic acid, iturin, or other antimicrobials play a role in the antagonistic activity of Pf 2-79 or *Bacillus* YD1.

In a previous study *(13), Salmonella* ceased to grow when microbial density on the surfaces of pepper disks reached the maximum of 10^9 CFU per disk. Application of an excessive level of antagonist would accelerate the achievement of this microbial density limit and consequently deplete the space or nutrients required for the growth of existing or incoming pathogens. Regardless of the initial level of antagonist (3.7 to 6.7 log CFU per disk), final populations of each antagonist reached the maximum of approximately 9 log CFU per disk on pepper disks after incubation of disks at 20°C for 2 days. Competitive exclusion *(24)* thus is another possible mechanism by which Pf 2-79 or *Bacillus* YD1 inhibited the growth of pathogens on pepper disks.

The data presented here indicate that the effectiveness of biological intervention is greatly affected by the ratio of antagonist to pathogen in the culture (Tables 3 and 4). Inoculation of pepper disks with Pf 2-79 or *Bacillus* YD1 at 5 to 6 log CFU per disk is sufficient to reduce the growth of four major pathogens on pepper disks by 3 to 4 log units. The initial pathogen level on pepper disks tested in this study (approximately 3 to 4 log CFU per disk) was much higher than that expected on naturally contaminated alfalfa seeds (less than 1 most probable number per 10 g of seed) *(19).* No report on the actual level of pathogens on naturally contaminated produce has been published. Provided that the contamination level does not exceed 3 to 4 log CFU/g of tissue, application of Pf 2-79 or *Bacillus* YD1 at 5 to 6 log CFU/g as used in this study should be sufficient to provide adequate protection against the growth of pathogens on fresh produce to a clinically significant level.

ACKNOWLEDGMENTS

I am grateful to Mr. Larry Revear for technical assistance and to Drs. Brendan Niemira and James Smith for critical reviews during the preparation of this manuscript.

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